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LETTERS

A conserved ubiquitination pathway determines longevity in response to diet restriction

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Dietary restriction extends longevity in diverse species, suggesting that there is a conserved mechanism for nutrient regulation and prosurvival responses[1]. Here we show a role for the HECT (homologous to E6AP carboxy terminus) E3 ubiquitin ligase WWP-1 as a positive regulator of lifespan in *Caenorhabditis elegans* in response to dietary restriction. We find that overexpression of *wwp-1* in worms extends lifespan by up to 20% under conditions of *ad libitum* feeding. This extension is dependent on the FOXA transcription factor *pha-4*, and independent of the FOXO transcription factor *daf-16*. Reduction of *wwp-1* completely suppresses the extended longevity of diet-restricted animals. However, the loss of *wwp-1* does not affect the long lifespan of animals with compromised mitochondrial function or reduced insulin/IGF-1 signalling. Overexpression of a mutant form of WWP-1 lacking catalytic activity suppresses the increased lifespan of diet-restricted animals, indicating that WWP-1 ubiquitin ligase activity is essential for longevity. Furthermore, we find that the E2 ubiquitin conjugating enzyme, UBC-18, is essential and specific for diet-restriction-induced longevity. UBC-18 interacts with WWP-1 and is required for the ubiquitin ligase activity of WWP-1 and the extended longevity of worms overexpressing *wwp-1*. Taken together, our results indicate that WWP-1 and UBC-18 function to ubiquitinate substrates that regulate diet-restriction-induced longevity.

HECT E3 ligases promote the ubiquitination of proteins that are essential in a variety of cellular events. The mammalian WWP1, WWP2 and ITCH family of WW domain HECT ligases (WWP ligases) were initially identified in a search for new proteins containing WW domains, which are modular protein-interaction domains recognizing short proline motifs in their partners[2]. WWP ligases have an amino-terminal C2 domain, which is a phospholipid membrane interaction motif, followed by four WW domains. To identify cellular pathways in which WWP E3 ligases are required, we have taken advantage of *C. elegans* as a model organism, which contains a single HECT WWP E3 ligase orthologue, *wwp-1* (Y65B4BR.4). Disruption of *wwp-1* using RNA interference (RNAi) yields a lethal phenotype late in embryogenesis characterized by abnormal embryogenesis despite normal cell proliferation[3]. The *wwp-1*(*ok1102*) mutant allele has a partially penetrant embryonic lethal phenotype[4]. Independent of the early developmental function of *wwp-1*, we found that the loss of *wwp-1* decreased stress resistance during adulthood (Supplementary Fig. 1a, b, e), leading us to investigate a possible role in longevity. Loss of *wwp-1* function by RNAi or mutation reduced lifespan at 25 °C (Supplementary Fig. 2a, b), but not at 20 °C (Supplementary Fig. 3a, b), consistent with a role for *wwp-1* in stress resistance. To investigate whether increased expression of *wwp-1* extended longevity in N2 (wild-type) worms, we created stable transgenic lines that express an N-terminal green fluorescent protein (GFP)–WWP-1 fusion protein, under the control of the endogenous *wwp-1* promoter in which expression of *wwp-1* messenger RNA is increased by approximately 50% (Supplementary Fig. 4). The overexpressing *wwp-1* transgenic lines (GFP::WWP-1) lived up to 20% longer than controls expressing GFP under the same promoter (Fig. 1a), indicating that *wwp-1* is a positive regulator of lifespan.

When diet is restricted, lifespan is extended in diverse species, suggesting that there is a conserved mechanism for nutrient regulation of ageing. Dietary restriction in worms can be reproduced genetically using *eat-2*(*ad1116*) mutant worms[5,6]. Reduced levels of *wwp-1* completely suppressed the extended longevity of *eat-2* mutant animals (Fig. 1b). The suppression of diet-restriction-extended lifespan by *wwp-1* depletion is unlikely to be due to increased food intake, because no difference in pharyngeal pumping rates with loss or knockdown of *wwp-1* in N2 or *eat-2*(*ad1116*) worms was observed (Supplementary Table 4).

We tested whether the loss of *wwp-1* suppressed the extended longevity of diet-restricted animals by reduced food intake imposed by bacterial dilution in liquid culture. N2 animals exhibited a bell-shaped curve for lifespan in response to varying bacterial concentrations (Fig. 1c)[7–9]. The lifespan of N2 animals grown under diet-restricted conditions was more than double that of animals fed *ad libitum* (Fig. 1c, d). In contrast, the lifespan of *wwp-1*(*ok1102*) mutant worms across the entire food concentration range did not change noticeably (Fig. 1c, d), indicating that WWP-1 has an essential role in regulating the response to nutrient intake and longevity. Similar results were seen using another method of dietary restriction, solid-plate dietary restriction[10] (Supplementary Fig. 5). To determine whether dietary restriction could affect *wwp-1* expression, we used quantitative PCR (qPCR) to quantify *wwp-1* mRNA and found no difference in *wwp-1* expression in animals grown under diet-restricted and *ad libitum* conditions (Supplementary Fig. 6). Furthermore, expression of a GFP::WWP-1 transgene partially rescued the suppression of dietary restriction longevity in *wwp-1*(*ok1102*) mutants (Supplementary Figs 4 and 7). Because the loss of *wwp-1* prevented the extension of lifespan of animals grown using three different diet-restriction methods, we conclude that *wwp-1* is essential for the increased longevity response to dietary restriction.

The FOXA transcription factor PHA-4 is required to specifically mediate diet-restriction-induced longevity in *C. elegans*[8]. RNAi reduction of *pha-4* suppressed the increased longevity of worms overexpressing *wwp-1* (Fig. 1e), but not when these worms were fed bacteria expressing double-stranded (ds)RNA against *daf-16*—the forkhead transcription factor required for the increased longevity due to reduced insulin/IGF1 signalling[11,12] (Fig. 1f). Mutations in the iron sulphur component of complex III, *isp-1*, increase longevity by reducing mitochondrial function[13–15]. *wwp-1* RNAi did not suppress the extended lifespan of *isp-1*(*qm150*) mutant animals (Fig. 1g), and

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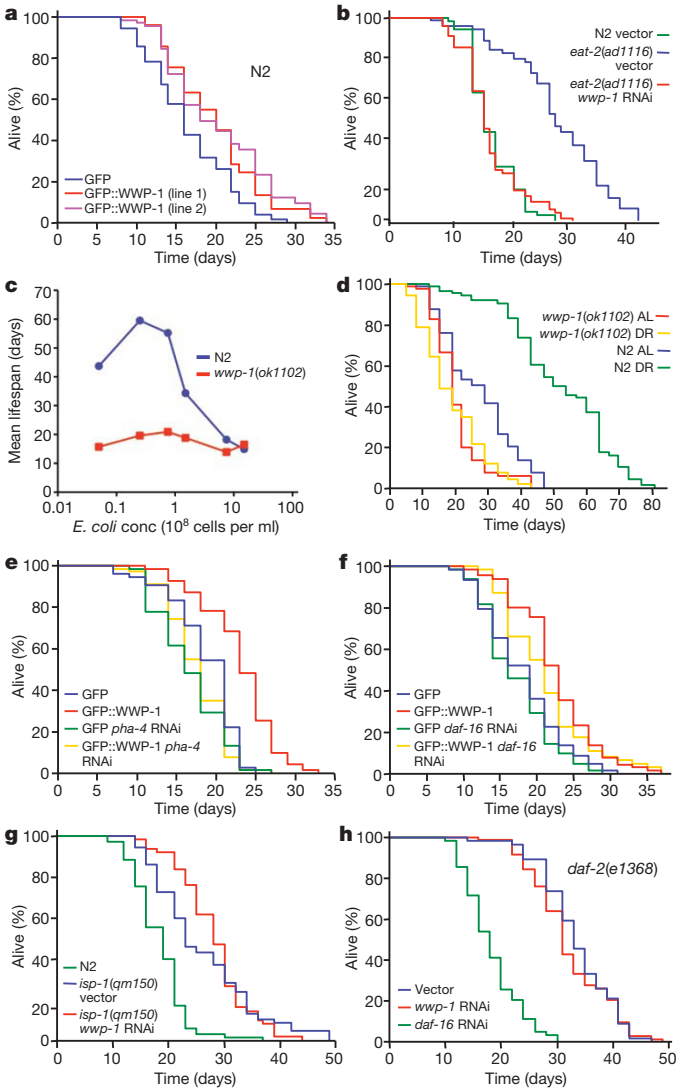

Figure 1 | *wwp-1* is required and specific for the extension of lifespan by dietary restriction. Lifespan values are given in Supplementary Tables 1 and 2. Two-way analysis of variance (ANOVA) results are presented in Supplementary Table 7. Supplementary Fig. 15a, c shows data confirming specific knockdown of *wwp-1* expression by RNAi. **a**, Two independent *wwp-1* overexpressing strains (GFP::WWP-1) can extend longevity compared to control worms expressing GFP. **b**, Lifespan analysis of *eat-2*(*ad1116*) mutant animals fed bacteria expressing *wwp-1* dsRNA or control vector. **c**, Lifespans of N2 and *wwp-1*(*ok1102*) mutant worms grown in S basal buffer with different *E. coli* concentrations. **d**, Lifespan analysis of N2 and *wwp-1*(*ok1102*) mutant worms grown in diet-restricted (DR) or *ad libitum* (AL) *E. coli* concentrations. **e**, **f**, Lifespan analysis of *wwp-1* overexpressing worms (GFP::WWP-1) or a control line fed bacteria expressing *pha-4* dsRNA (**e**), or *daf-16* dsRNA (**f**). **g**, **h**, Lifespan analysis of *isp-1*(*qm150*) (**g**) and *daf-2*(*e1368*) (**h**) fed bacteria expressing *wwp-1* dsRNA or control vector.

had only minor suppressive effects on lifespan extension of another mitochondrial mutant, *clk-1*(*qm30*), and in *cyc-1* RNAi-treated worms (Supplementary Fig. 8). Partial loss of function mutations in the insulin/IGF-1 receptor homologue, DAF-2, increase lifespan in a *daf-16*-dependent, *pha-4*-independent manner[8,16]. RNAi depletion of *wwp-1* had no effect on the long lifespan of *daf-2* mutant animals (Fig. 1h and Supplementary Fig. 9a). Our results indicate that loss of *wwp-1* does not make animals sick, but rather specifically regulates the response to dietary restriction that results in extended longevity.

Ubiquitination by HECT ligases requires the intermolecular transfer of ubiquitin from an associated E2 to the E3 ligase, before transfer to a lysine in the target protein[17]. These transfers depend on the formation of a thioester bond between ubiquitin and a conserved cysteine in the HECT domain. Mutation of this cysteine renders HECT ligases catalytically inactive, and the mutants act as dominant negatives *in vivo*[18]. We established an *in vitro* ubiquitination assay for WWP-1 ligase activity using *C. elegans* embryo extract as a source of substrates. In the presence of extract, bacterially expressed glutathione *S*-transferase (GST)–WWP-1 had very robust ligase activity, which was abolished by mutation of the catalytic cysteine (C762A) of WWP-1 (Fig. 2a). We then compared the longevity of *eat-2*(*ad1116*) transgenic animals that overexpress a GFP–WWP-1(C762A) fusion protein driven by the *wwp-1* promoter to a control line expressing GFP under the same promoter. Two independent *eat-2*(*ad1116*) transgenic lines expressing the dominant negative *wwp-1* had a significantly shorter lifespan, comparable to wild-type animals (Fig. 2b). Therefore, the ubiquitin ligase activity of WWP-1 is essential for diet-restriction-induced longevity.

UBC-18 is a putative E2 that regulates pharyngeal morphogenesis during early embryonic development[19–21]. UBC-18 is homologous to human UBCH7 (also known as UBE2L3), and similar to *Saccharomyces cerevisiae* Ubc5 and Ubc4 (ref. 21). Recently, a two-hybrid screen using UBC-18 as a bait identified WWP-1 and the RING finger E3 ligases ARI-1 and F56D2.2 as UBC-18 interactors[19]. Unlike *ubc-18* and *ari-1* dsRNA, inactivation of *wwp-1* by dsRNA treatment failed to produce a pharynx-unattached phenotype in *pha-1*(*e2123*) animals, suggesting that WWP-1 may function with UBC-18 to ubiquitinate targets not involved in pharyngeal development[19]. Consistent with this, RNAi of either *ari-1* or F56D2.2 did not affect the long lifespan of the *eat-2* mutant animals (Supplementary Fig. 10).

We found that UBC-18 is indeed a functional E2. UBC-18 formed a thiol ester bond with ubiquitin (Fig. 3a), and recombinant WWP-1 ubiquitin ligase activity required UBC-18 and E1 *in vitro* (Fig. 3b). Extracts prepared from worms mutant for *ubc-18*, *ubc-18*(*ku354*)[21], greatly reduced WWP-1-dependent ubiquitin ligase activity, which was restored by the addition of recombinant UBC-18 (Fig. 3b). Finally, we confirmed that UBC-18 and WWP-1 associate *in vitro* (Fig. 3c).

We tested whether *ubc-18* was essential for diet-restriction-induced longevity. Like *wwp-1*, *ubc-18* has a role in stress resistance in *C. elegans* (Supplementary Fig. 1c–e). However, we found that overexpression of *ubc-18* was unable to extend lifespan in *C. elegans* (Supplementary Fig. 11). Possibly, UBC-18 is not limiting for WWP-1 function in lifespan. Loss of *ubc-18* function reduced lifespan at 25 °C (Supplementary Fig. 2c), but only slightly at 20 °C (Supplementary

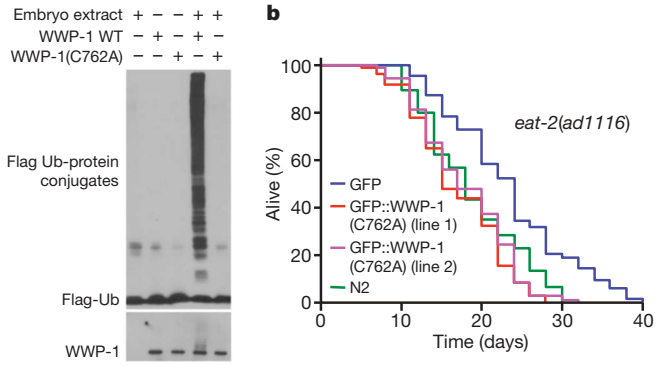

Figure 2 | WWP-1 ubiquitin ligase activity is essential for diet-restriction-induced longevity. a, Mutation of the conserved catalytic cysteine of WWP-1 abolishes ubiquitin (Ub) ligase activity. *In vitro* ubiquitination assay of recombinant wild-type (WT) WWP-1 or mutant WWP-1(C762A) using *C. elegans* embryo extract. **b**, *eat-2*(*ad1116*) mutant worms expressing a dominant negative *wwp-1*(C762A) have significantly shorter lifespans than control worms expressing GFP. Lifespan values are given in Supplementary Table 1.

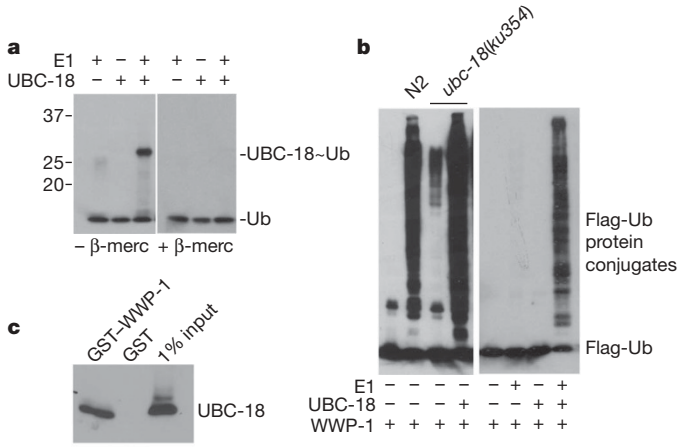

Figure 3 | WWP-1 exhibits ubiquitin ligase activity in a UBC-18 dependent manner *in vitro*. **a**, UBC-18 forms thiol-sensitive adducts with ubiquitin. *In vitro* ubiquitin (Ub) conjugation reaction in which samples were subjected to SDS–PAGE with or without β-mercaptoethanol (β-merc). **b**, UBC-18 is essential for ubiquitin ligase activity *in vitro*. Left panel, *in vitro* ubiquitination assay of wild-type WWP-1 using N2 or *ubc-18* mutant (*ubc-18*(*ku354*)) embryo extract. Recombinant UBC-18 was added in the last lane. Right panel, *in vitro* ubiquitination assay using purified components. **c**, GST pull-down assay in which GST–WWP-1 (or GST alone) bound to glutathione-agarose beads was incubated with cell lysates expressing *ubc-18*.

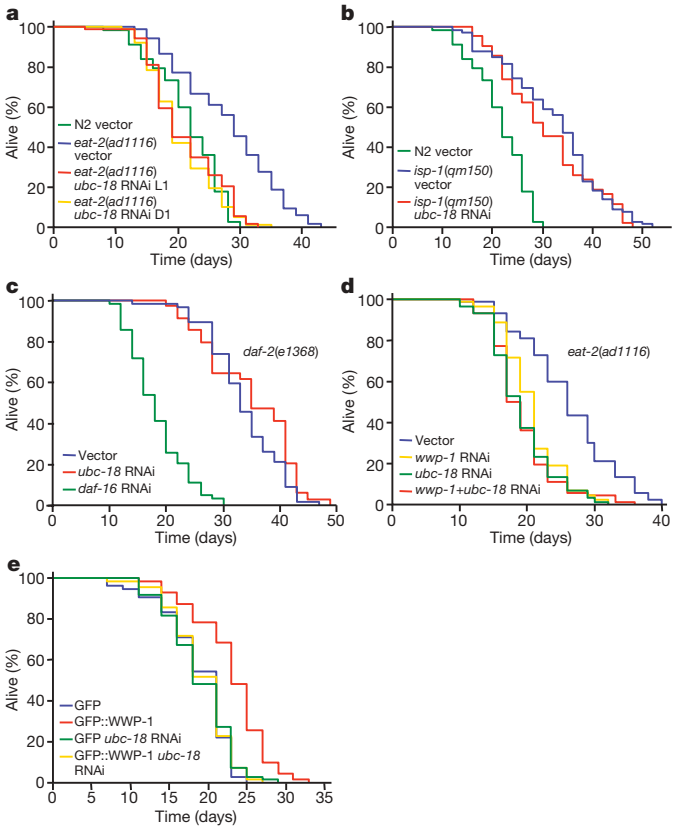

Figure 4 | WWP-1 and UBC-18 function together to regulate diet-restriction-induced longevity. Lifespan values are given in Supplementary Table 1. Two-way ANOVA results are presented in Supplementary Table 7. Knockdown of *ubc-18* expression by RNAi is shown in Supplementary Fig. 15d. **a**, Lifespan analysis of *eat-2*(*ad1116*) mutant worms fed bacteria expressing *ubc-18* dsRNA or control vector initiated after hatching of eggs (L1) or day 1 adults (D1). **b**, **c**, Lifespan analysis of *isp-1*(*qm150*) (**b**) and *daf-2*(*e1368*) (**c**) fed bacteria expressing *ubc-18* dsRNA or control vector. **d**, Lifespan analysis of *eat-2*(*ad1116*) mutant animals fed bacteria expressing *wwp-1* dsRNA and vector (*wwp-1* RNAi), *ubc-18* dsRNA and vector (*ubc-18* RNAi), *wwp-1* and *ubc-18* dsRNA (*wwp-1* + *ubc-18* RNAi), or control vector. **e**, Lifespan analysis of *wwp-1* overexpressing worms (GFP::WWP-1) or control worms (GFP) fed bacteria expressing *ubc-18* dsRNA or control vector.

Fig. 3c, d). However, RNAi depletion of *ubc-18* completely suppressed the increased longevity of *eat-2* mutants (Fig. 4a). This decreased lifespan is unlikely to be due to impaired pharynx function, because RNAi was initiated at the L1 stage when the pharynx is completely developed, and *ubc-18* RNAi initiated at the first day of adulthood also suppressed the increased longevity of *eat-2*(*ad1116*) animals. Like *wwp-1* depletion, we did not see a difference in pharyngeal pumping rates with loss of *ubc-18* (Supplementary Table 4), and RNAi depletion of *ubc-18* had no effect on the long lifespan of *isp-1*(*qm150*) (Fig. 4b) or *daf-2* mutant animals (Fig. 4c and Supplementary Fig. 9b). Moreover, epistasis analysis of *wwp-1* and *ubc-18* indicated that the combined knockdown of both genes by RNAi in *eat-2*(*ad1116*) animals did not shorten lifespan any further than RNAi of either single gene (Fig. 4d). Finally, knockdown of *ubc-18* suppressed the extended lifespan of *wwp-1* overexpressing animals (Fig. 4e).

In summary, the UBC-18–WWP-1 complex functions to specify the longevity response of diet-restricted animals. Because E2s often function with several E3s, it is surprising to find that *ubc-18* was not only essential, but also specific for the response to dietary restriction. M7.1 (also known as UBC-2 and LET-70) is most homologous to UBCH5 (also known as UBE2D1), a mammalian E2 that associates with HECT ubiquitin ligases. Unlike *ubc-18*, loss of *ubc-2* did not specifically suppress the extended longevity of *eat-2* mutants and resulted in general sickness of animals (Supplementary Fig. 12). The other E3s that interact with UBC-18 may be dedicated instead to the developmental function of UBC-18, as is the case for ARI-1 (ref. 19). It is interesting that expression of WWP-1 and UBC-18 is observed in several neurons localized in the head and tail of adult animals (Supplementary Fig. 13), because many recent studies in *C. elegans* and *Drosophila* suggest that signals derived from the nervous system can control longevity[22–25]. Although it is intriguing to speculate that a few key neuronal cells in the nervous system are the site of action of WWP-1–UBC-18 to regulate longevity, expression is not confined to a few neurons, as is the case for the dietary restriction regulator, SKN-1B[7]. Furthermore, expression of WWP-1–UBC-18 is found in intestinal cells, another site where longevity cues are expressed in the worm[26].

Because several transcription factors have been identified as targets for the mammalian orthologues of *wwp-1* (ref. 17), we investigated whether WWP-1 may target one of the two transcription factors essential for dietary restriction longevity in the worm: PHA-4 and SKN-1B[7,8]. The genetic epistasis analysis of *ubc-18/wwp-1* suggested that PHA-4 may be a target for ubiquitination. Using our *in vitro* ubiquitination assay for WWP-1, we were unable to detect ubiquitinated conjugates for either PHA-4 or SKN-1B in a purified system (Supplementary Fig. 14). Recently it has been shown that *pha-4* and the CeTor (also known as *let-363*) pathway antagonize one another to regulate longevity in adults[27]. Our results might suggest that *wwp-1* may feed into the CeTor pathway as well. The identification of the targets of UBC-18–WWP-1 is needed to allow precise placement of this complex in the diet-restriction pathway.

Our study uncovers for the first time, to our knowledge, a role of the ubiquitin pathway in longevity in response to dietary restriction. Given the strong conservation of *wwp-1* with mouse and human WWP1, an attractive hypothesis is that the mammalian orthologue will also be critical for diet-restriction-induced longevity. A detailed understanding of the pathways that mediate the benefits of dietary restriction may lead to new therapies for age-related diseases.

METHODS SUMMARY

***Caenorhabditis elegans* methods.** The *wwp-1* mutant strain was generated by backcrossing RB1178 (*wwp-1*(*ok1102*)) to N2 three times (Supplementary Fig. 15b). Nematodes were handled using standard methods[28].

Lifespan analysis. Lifespan analyses were performed as described[29]. Bacterial dilution diet-restricted lifespans were performed as described[8] with the following modifications: synchronized populations of eggs were hatched and grown at 20 °C on NG agar plates containing OP50 *E. coli* until the L4 larval stage when they were transferred to plates of OP50 containing 100 μg ml^{-1} FUDR. At day 1 of adulthood, worms were transferred into liquid culture. All lifespans were performed at 20 °C unless noted.

Protein extraction for *in vitro* ubiquitination assay. *Caenorhabditis elegans* embryos were isolated using an alkaline hypochlorite solution from gravid N2 worms grown at 20 °C[30]. The embryos were resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 0.75 mM EDTA, 1.5 mM dithiothreitol (DTT), 2.5 mM PMSF, 1 μg ml^{-1} aprotinin, 1 μg ml^{-1} leupeptin, 1 μg ml^{-1} pepstatin A) and homogenized with 30 strokes in a Dounce homogenizer. The extract was centrifuged 15,000*g* at 4 °C and stored at −70 °C.

***In vitro* ubiquitination assay.** The ubiquitination assay was carried out by incubating 1 μg Flag-tagged ubiquitin, 0.5 μg GST–WWP-1 (wild-type or C762A mutant), 0.1 μg UBC-18 and 15–20 μg embryo extract in 30 μl reaction buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM DTT and 5 mM ATP) for 1 h at 30 °C. The reaction was stopped with sample buffer and run on denaturing protein gels. Ubiquitinated substrates were identified by anti-Flag (M2, Sigma) immunoblotting. For *in vitro* ubiquitination assays using purified components, similar conditions were administered except 0.5 μg UBC-18 and 1 μg E1 were used. To measure UBC-18 ubiquitin conjugation, a similar reaction was performed and the reaction was stopped with sample buffer lacking β-mercaptoethanol.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions A.C.C. designed the experiments and analysed the data. A.C.C. and Z.L. performed the experiments. A.D. and T.H. supervised the design and data interpretation. The manuscript was written by A.C.C. and edited by A.D. and T.H. All authors discussed the results and commented on the manuscript.

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METHODS

Statistical analysis. JMP IN 5.1 software was used to determine means and percentiles. *P* values were calculated using the log-rank (Mantel-Cox) method. To assess differences in mortality between N2 and *wwp-1*(*ok1102*) strains in diet-restriction lifespans, a Cox proportional hazards regression model was generated in JMP IN 5.1 software. Two-way ANOVA was performed using Prism Version 5.0 software. Mean lifespan ± s.e.m. and *n* of all experiments presented in Supplementary Tables 1 and 5 were considered with strain and RNAi treatment as the factors.

Creation of WWP-1 and UBC-18 constructs for expression in *C. elegans*. To construct the plasmid expressing GFP::WWP-1 driven by the *wwp-1* endogenous promoter, sequences 644 bases upstream of the *wwp-1* coding region were amplified from genomic DNA by PCR and inserted upstream of GFP sequences in the worm expression vector pPD95.77. This construct (*Pwwp-1*::GFP), which expresses GFP under the control of the endogenous *wwp-1* promoter, was used as a control. To generate an N-terminal GFP-fusion construct, point mutagenesis (Stratagene) was performed on the GFP construct to eliminate the stop codon in the GFP complementary DNA. Full-length *wwp-1* cDNA was amplified from a first-strand cDNA from N2 worms by PCR, and inserted into the N-terminal GFP-fusion construct containing the *wwp-1* endogenous promoter downstream and in frame with the GFP sequence. This construct (*Pwwp-1*::GFP::WWP-1) expresses GFP–WWP-1 fusion protein under the control of the *wwp-1* promoter. To generate *wwp-1*(C762A) dominant negative mutants, point mutagenesis was performed on the *Pwwp-1*::GFP::WWP-1 construct to generate *Pwwp-1*::GFP::WWP-1(C762A). Primers for the *wwp-1* promoter: forward, 5′-GCT CTAGACTTGTTTCCTGATGACCTTG-3′; reverse, 5′-CGGGATCCTCGATC ATGAAACTGGCTG-3′. Primers for *wwp-1* cDNA: forward, 5′-ATGGCTCG TAATGAACCATCATCTCAGCAG-3′; reverse, 5′-CTACTCGTTTCCAAATCC TTCCGTCATCTC-3′. To construct the plasmid expressing GFP driven by the *ubc-18* endogenous promoter (*Pubc-18*::GFP), sequences 911 bases upstream of the *ubc-18* coding region were amplified from genomic DNA by PCR and inserted upstream of GFP sequences in the worm expression vector pPD95.67. To construct the plasmids expressing UBC-18 driven by the *ubc-18* promoter, sequences from 911 bases upstream of the *ubc-18* coding region to the stop codon of the *ubc-18* coding region were amplified from genomic DNA by PCR and inserted upstream of the GFP sequence in the worm expression vector pPD95.77. Primers for *ubc-18* promoter: forward, 5′-GATCTTTC TACTGTAGAC-3′; reverse, 5′-CGTTGATATAAACGCTCTAG-3′. Primers to generate *Pubc-18*::UBC-18::GFP: forward, 5′-GATCTTTCTACTGTAGAC-3′; reverse, 5′-GCGTATTCAGGCCGCTTTTC-3′. Primers to generate *Pubc-18*::UBC-18*:* forward, 5′-GATCTTTCTACTGTAGAC-3′; reverse, 5′-CTA TTCAGGCCGCCGCTTTTC-3′.

Generation of transgenic lines. Extrachromosomal array carrying transgenic strains were generated using standard microinjection methods[31]. For generation of N2 animals overexpressing *wwp-1*, *Pwwp-1*::GFP::WWP-1 plasmid DNA construct was mixed at 20 μg ml^{-1} with 80 μg ml^{-1} of pRF4(*rol-6*) construct[31]. *wwp-1*(*ok1102*) mutant animals overexpressing *wwp-1* were generated by crossing *wwp-1*(*ok1102*) mutants to *Pwwp-1*::GFP::WWP-1 males. For generation of *eat-2*(*ad1116*) worms overexpressing dominant negative WWP-1(C762A), *Pwwp-1*::GFP::WWP-1(C762A) plasmid DNA was mixed at 30 μg ml^{-1} with 80 μg ml^{-1} of pRF4 construct. Worms used as controls against *wwp-1* overexpressing strains contained 30 μg ml^{-1} *Pwwp-1*::GFP expressing construct injected with 30 μg ml^{-1} pRF4. For generation of N2 animals overexpressing *ubc-18*, *Pubc-18*::UBC-18::GFP or *Pubc-18*::UBC-18 plasmid DNA construct was mixed at 75 μg ml^{-1} with 75 μg ml^{-1} pRF4 construct. Worms used as controls against *ubc-18* overexpressing strains contained 75 μg ml^{-1} *Pubc-18*::GFP expressing construct injected with 75 μg ml^{-1} pRF4 construct. Transgenic F_1 progeny were selected on the basis of roller phenotype. Individual transgenic F_2 animals were isolated to establish independent lines.

GFP and DiI localization. Paralysed transgenic animals were assayed for GFP expression at ×10 and ×63 magnification using a Leica 6000B digital microscope. Images were acquired using Leica FW4000 and AF6000 software. For DiI assays, worms were incubated with 5 μg ml^{-1} DiI in M9 at room temperature for 2 h, then transferred to fresh plates for 1 h to destain, and examined under the fluorescence microscope. Intestinal granule autofluorescence was viewed with the 4,6-diamidino-2-phenylindole (DAPI) filter.

Stress assays. For heat-shock assays, eggs were grown on plates seeded with various RNAi treatments to D1 adulthood. Worms were transferred to plates without food and heat shocked at 35 °C and were checked every 2 h for viability. Paraquat (methyl viologen, Sigma) assays were performed as described[29]. For all stress assays a representative experiment from at least two independent experiments is presented.

Pumping-rate assays. Pumping rates were determined by counting pumps of the terminal pharyngeal bulb for 1-min intervals. For each condition, worms were treated with bacteria expressing dsRNA since hatching. The pumping rates of ten D1 adult worms per condition were measured and averaged.

Solid-plate dietary restriction. Lifespans were performed as described[10] with the following modifications: adult worms are transferred every 2–3 days to freshly seeded plates with restrictive amounts of bacteria (5×10^7 to 5×10^{10} bacteria per ml) starting at day 5 of adulthood.

RNA isolation, RT–PCR and quantitative RT–PCR. Total RNA was isolated from asynchronous populations of worms and extracted using Trizol reagent (GIBCO). cDNA was created using Superscript II RT (Invitrogen) and oligo dT primers. For quantitative PCR with reverse transcription (qRT–PCR) experiments, approximately 2,000 young adult worms with roller phenotype were hand picked from extrachromasomal array carrying transgenic lines grown at 20 °C. To determine how dietary restriction could affect *wwp-1* expression, bacterial dilution diet-restriction cultures were performed as described above but at a larger scale to collect 4,000 worms per sample. Total RNA was extracted using Trizol reagent and cDNA was created using Quantitec Reverse Transcriptase kit (Qiagen). SYBRgreen real-time qPCR experiments were performed as described in the manual using ABI Prism 7900 HT (Applied Biosystems). Quantitative PCR primers: *wwp-1* forward, 5′-AAGAAGCGCAGGAGTACGAG-3′; *wwp-1* reverse, 5′-ATTGATCGAAACGCATCTCC-3′; *pmp-3* forward, 5′-GTTCCC GTGTTCATCACTCAT-3′; *pmp-3* reverse, 5′-ACACCGTCGAGAAGCTG TAGA-3′.

Recombinant proteins and GST pull-down experiments. Full-length *wwp-1* cDNA (wild-type and C762A mutant) or *ubc-18* cDNA was inserted into pGEX-KG bacterial expression vector. Full-length *skn-1b* and *pha-4* cDNA were inserted into pHis8 bacterial expression vector. His purification was performed using Talon Metal Affinity Resin (Clontech). GST and His purifications were performed according to the manufacturer's instructions. Flag-tagged ubiquitin has been previously described[32,33]. For active protein, the GST tag on UBC-18 was removed by thrombin cleavage, and the supernatant was incubated with benzamidine Sepharose-4B beads (GE Healthcare) for 15 min at room temperature to remove thrombin. Full-length *ubc-18* cDNA was inserted into pcDNA6/Myc-His mammalian expression vector to generate a Myc–His fusion protein. GST-pull-down experiments were performed using 500 μg NIH3T3 lysates expressing *ubc-18* by transient transfection (Effectene, Qiagen). Lysates were prepared as previously described[34]. Lysates were incubated with GST–WWP-1 or GST bound to glutathione agarose beads for 1.5 h. Beads were washed four times with lysis buffer and analysed my SDS–PAGE. UBC-18 was detected by immunoblotting with an anti-Myc (9E10) antibody.

***In vitro* ubiquitination assay for PHA-4 and SKN-1B.** The ubiquitin ligase assay for PHA-4 and SKN-1B was carried out by incubating 1 μg Flag-tagged ubiquitin, 0.5 μg GST–WWP-1 (wild-type or C762A mutant), 0.5 μg UBC-18, 0.1 μg His–E1 and ~1 μg His–PHA-4/SKN-1B in 30 μl reaction buffer for 1 h at 30 °C. UBC-18 was preincubated with 1 mM PMSF to inhibit any residual thrombin activity. The reaction was stopped with sample buffer and run on a protein gel under denaturing conditions. PHA-4 and SKN-1B were detected by immunoblotting with a monoclonal anti-poly Histidine antibody (Sigma).

RNAi treatment. RNAi-treated strains were fed *E. coli* (HT115) containing an empty vector or *E. coli* expressing dsRNA against the gene of interest. The *wwp-1* and *pha-4* RNAi clones were from J. Ahringer's RNAi library[35]. RNAi clones against *ubc-18*, *ari-1* and F56D2.2 were from M. Vidal's RNAi library[36]. RNAi clones against *daf-16*, *daf-2* and *cyc-1* have been previously described[37,38]. Double RNAi experiments were performed as described[39].

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